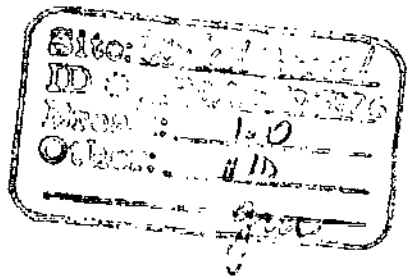
TABLE 2. Preliminary Summary Of In Vitro Bioassay Results

Sample	ID	Pb in <250u bulk soil mg/kg	mass soil (g)	calc Pb #1	ICP Pb (mg/l)	solution amt (l)	% Relative Pb Bioavailability
UZ-1	EPA 7 C#3	7043	1.00047	7.05	52.48	0.1	74
UZ-2	EPA 136a C#3	2496	1.00052	2.50	18.08	0.1	72
UZ-3	EPA 21 C#2	5113	1.0004	5.12	43.27	0.1	85
UZ-4	EPA 54 C#1	2285	1.00097	2.29	18.05	0.1	79
UZ-5	EPA 48 C#2	861	1.00087	0.86	7.42	0.1	86
UZ-6	EPA 223 C#1	3383	1.00041	3.38	22.54	0.1	67



The In-Vitro Method

University of Colorado

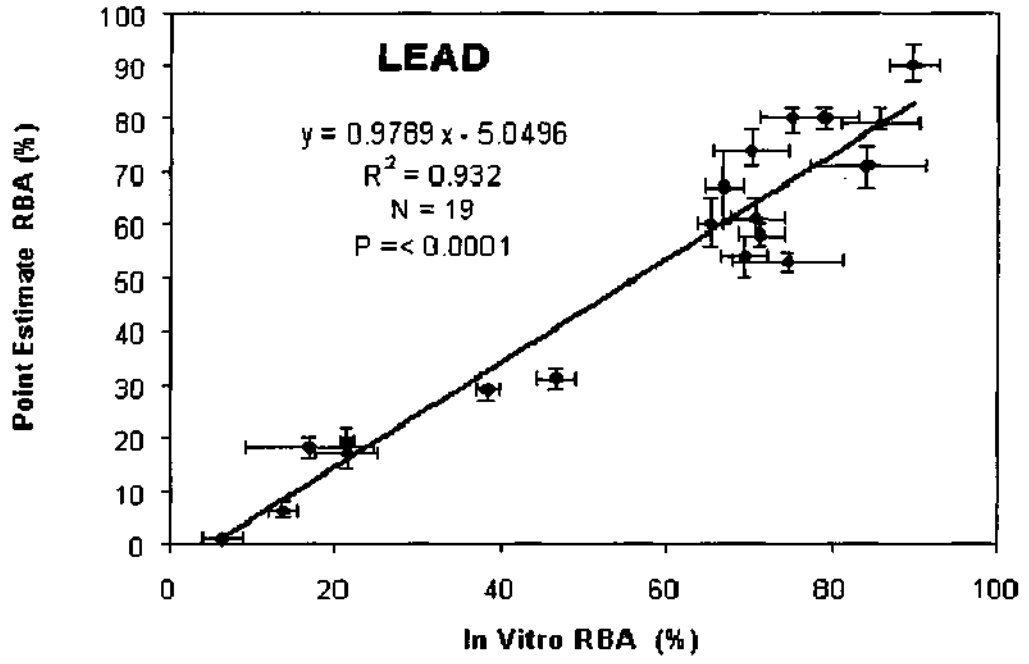
Relative Bioavailability Leaching Procedure

Standard Operating Procedure

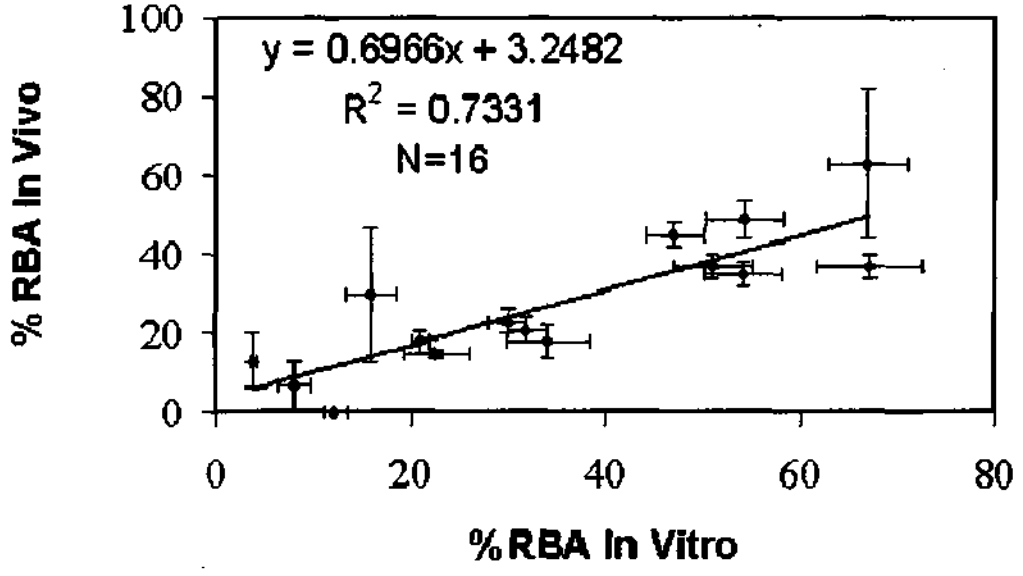
1.0 Purpose

An increasingly important property of contaminated media found at environmental sites is the bioavailabilty of individual contaminants. Bioavailability is the fraction of a contaminant that is absorbed by an organism via a specific exposure route. Many animal studies have been conducted to experimentally determine oral bioavailability of individual metals, particularly lead and arsenic. During the period 1989-97, a juvenile swine model developed by USEPA Region VIII was used to predict the relative bioavailability of lead and arsenic in approximately 20 substrates (Weis and LaVelle 1991; Weis et al. 1994). The bioavailability determined was relative to that of a soluble salt (i.e. lead acetate trihydrate or sodium arsenate). The tested media had a wide range of mineralogy, and produced a range of lead and arsenic bioavailabilty values. In addition to the swine studies, other animal models (e.g. rats and monkeys) have been used for measuring the bioavailabilty of lead and arsenic from soils.

Several researchers have developed in vitro tests to measure the fraction of a chemical solubilized from a soil sample under simulated gastrointestinal conditions. The in vitro tests consist of an aqueous fluid, into which the contaminant is introduced. The solution than solubilizes the media under simulated gastric conditions. Once this procedure is complete, the solution is analyzed for lead and/or arsenic concentrations. The mass of the lead and/or arsenic found in the filtered extract is compared to the mass introduced into the test. The fraction liberated into the aqueous phase is defined as the bioavailable fraction of lead or arsenic in that media. To date, for lead-bearing materials tested in the USEPA swine studies, this in vitro assay has correlated well ($R2 = 0.93$, $p = .0001$) with relative bioavailability. Arsenic has yet to be fully validated but shows a promising correlation with in vivo results.



ARSENIC



It has been postulated that a simplified in vitro method could be used to determine bioavailability of lead and arsenic. The method described in this SOP represents a simplified in vitro method, which is currently being subjected to a formal validation.

2.0 Scope

This procedure has been developed to test contaminated media in animal studies, to determine the correlation between in vitro and in vivo. Only samples from which mineralogy has been fully characterized by EMPA techniques and for which bioavailability results from acceptable animal studies are available have been used for this study. A total of 20 substrates have been tested in validating the relative bioavailability leaching procedure (RBLP).

3.0 Relevant Literature

Background on the development and validation of in vitro test systems for estimating lead and arsenic bioaccessibility can be found in; Ruby et al. (1993, 1996); Medlin (1972); Medlin and Drexler, 1997; Drexler, 1998; and Drexler et al., 2003.

Background information for the USEPA swine studies may be found in (Weis and LaVelle, 1991; Weis et al. 1994; and Casteel et al., 1997) and in the USEPA Region VIII Center in Denver, Colorado.

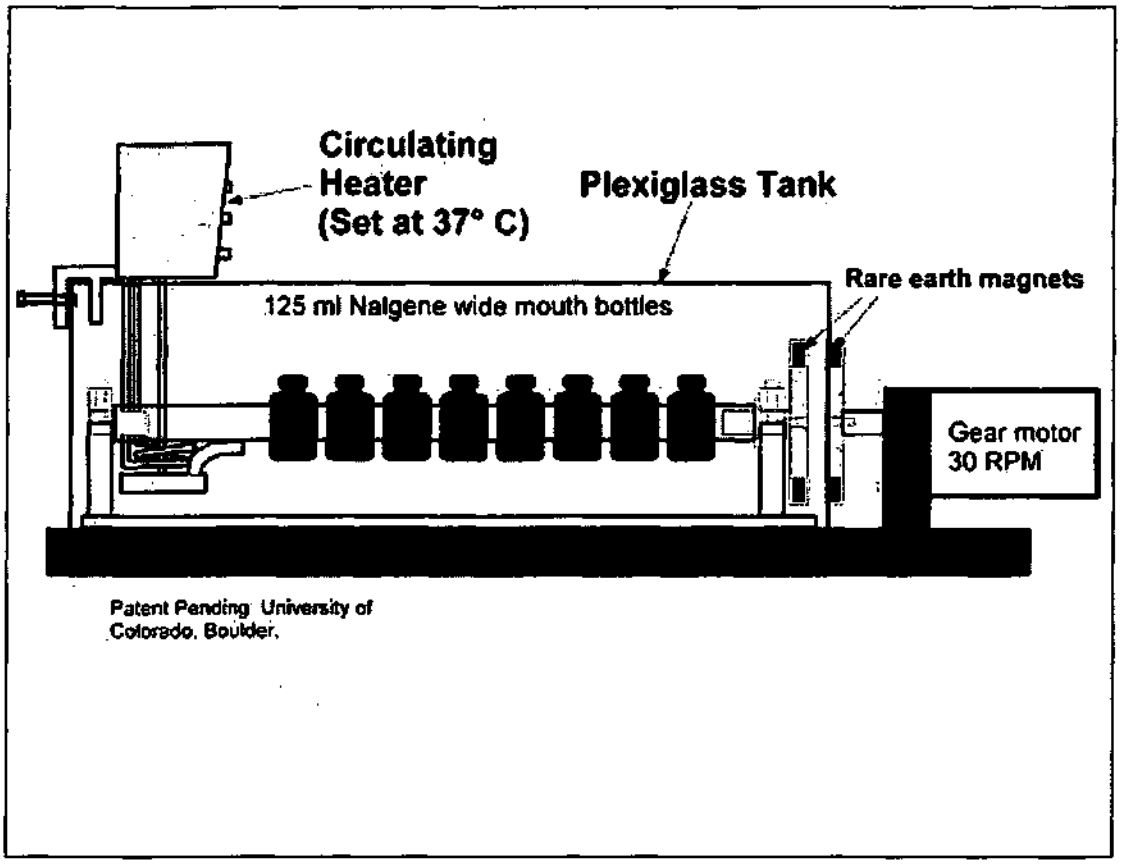
4.0 Sample Preparation

All media are prepared for the in vitro assay by first drying (<40 °C) all samples and then sieving to < 250 m m. The <250 micron size fraction was used because this is the particle size is representative of that which adheres to children's hands. Samples were thoroughly mixed prior to use to ensure homogenization. Samples are archived after the study completion and retained for further analysis for a period of six months unless otherwise requested. Prior to obtaining a subsample for testing in this procedure, each sample must be homogenized in its sample container by end-over-end mixing.

5.0 Apparatus and Materials

5.1 Equipment

The main piece of equipment required for this procedure is the extraction device illustrated in Figure 1. The device can be purchased from the Department of Geological Sciences, University of Colorado. For further information contact Dr. John W. Drexler, at (303) 492-5251 or drexlerj@spot.colorado.edu. The device holds ten 125 ml, wide-mouth high-density polyethylene (HDPE) bottles. These are rotated within a Plexiglas tank by a TCLP extractor motor with a modified flywheel. The water bath must be filled such that the extraction bottles remained immersed. Temperature in the water bath is maintained at 37 +/- 2 °C using an immersion circulator heater (Fisher Scientific Model 730).



The 125-ml HDPE bottles must have an airtight screw-cap seal (Fisher Scientific #02-893-5C), and care must be taken to ensure that the bottles do not leak during the extraction procedure.

5.2 Standards and Reagents

The leaching procedure for this method uses an aqueous extraction fluid at a pH value of 1.5. The pH 1.5 fluid is prepared as follows:

Prepare 2 L of aqueous extraction fluid using ASTM Type II demonized (DI) water. The buffer is made up in the following manner. To 1.9 L of DI water, add 60.06 g glycine (free base, reagent grade), and bring the solution volume to 2 L (0.4M glycine). Place the mixture in the water bath at 37 °C until the extraction fluid reaches 37 °C. Standardize the pH meter (one should use both a 2.0 and a 4.0 pH buffer for standardization) using temperature compensation at 37 °C or buffers maintained at 37 °C in the water bath. Add trace metal grade, concentrated hydrochloric acid (12.1N) until the solution pH reaches a value of 1.50 +/_ 0.05 (approximately 60 mL).

All reagents must be free of lead and arsenic, and the final fluid must be tested to confirm that lead and arsenic concentrations are less than one-fourth the project required detection limits (PRDLs) of 10 and 20 µg/L, respectively (e.g., less than 2 µg/L lead and 5µg/L

arsenic in the final fluid.

Cleanliness of all materials used to prepare and/or store the extraction fluid and buffer is essential. All glassware and equipment used to prepare standards and reagents must be properly cleaned, acid washed, and finally, triple-rinsed with demonized water prior to use.

6.0 Leaching Procedure

Add 1.00 +/- 0.5 g of test substrate (<250m m) to the bottle, ensuring that static electricity does not cause soil particles to adhere to the lip or outside threads of the bottle. If necessary, use an antistatic brush to eliminate static electricity prior to adding the media. Record the mass of substrate. When ready to begin the test-- measure 100 +/- 0.5 mL of the extraction fluid, using a graduated cylinder or auto pipette and transfer to the 125 mL wide-mouth HPDE bottles. Hand-tighten each bottle top and shake/invert to ensure that no leakage occurs, and that no media is caked on the bottom of the bottle.

Place the bottle into the modified TCLP extractor, making sure each bottle is secure and the lid(s) are tightly fastened. Fill the extractor with 125 mL bottles containing test materials or QA samples.

The temperature of the water bath must be 37 +/- 2 °C.

Turn on the extractor and rotate end-over-end at 30 +/- 2 rpm for 1 hour. Record the start time of rotation.

When extraction (rotation) is complete, immediately stop the extractor rotation and remove the bottles. Wipe them dry and place upright on the bench top.

Draw extract directly from the reaction vessel into a disposable 20 cc syringe with a Luer-Lok attachment. Attach a 0.45 µm cellulose acetate disk filter (25 mm diameter) to the syringe, and filter the extract into a clean 15 mL polypropylene centrifuge tube (labeled with sample ID) or other appropriate sample vial for analysis.

Record the time that the extract is filtered (i.e. extraction is stopped). If the total time elapsed is greater than 1 hour 30 minutes, the test must be repeated.

Measure the pH of the remaining fluid in the extraction bottle. If the fluid pH is not within +/_ 0.5 pH units of the starting pH, the test must be discarded and the sample reanalyzed as follows:

If the pH has changed more than 0.5 units, the test will be re-run in an identical fashion. If the second test also results in a decrease in pH of greater than 0.5 s.u. this will be recorded, and the extract filtered for analysis. If the pH has increased by 0.5 s.u. or more, the test must be repeated, but the extractor must be stopped at specific intervals and the pH manually adjusted down to pH of 1.5 with dropwise addition of HCl (adjustments at

5, 10, 15, and 30 minutes into the extraction, and upon final removal from the water bath { 60 min}). Samples with rising pH values might better be run following the method of Medlin, 1997.

Store filtered samples in a refrigerator at 4 °C until the are analyzed. Analysis for lead and arsenic concentrations must occur within 1 week of extraction for each sample.

Extracts are to be analyzed for lead and arsenic, as specified in EPA methods 6010B, 6020, or 7061A.

6.1 Quality Control/Quality Assurance

Quality Assurance for the extraction procedure will consist of the following quality control samples.

Bottle Blank-extraction fluid only run through the complete procedure at a frequency of 1 in 20 samples.

Duplicate sample-duplicate sample extractions to be performed on 1 in 10 samples.

Matrix Spike-a subsample of each material used will be spiked at concentrations of 10 mg/L lead and 1 mg/L arsenic and run through the extraction procedure (frequency of 1 in 10 samples).

National Institute of Standards and Testing (NIST) Standard Reference Material (SRM) 2711 will be used as a control soil. The SRM will be analyzed at a frequency of 1 in 20 samples.

Control limits and corrective actions are listed in Table 1.

	Analysis Frequency	**Control Limits**
Bottle blank	5% - 1:20	< 25 µ g/L lead
Blank spike *	5% - 1:20	85-115% recovery
Matrix spike *	10% - 1:10	75-125% recovery
Duplicate sample	10% - 1:10	+/- 20% RPD**
Control soil ***	5% - 1:20	+/- 10% RPD

* Spikes contained 10 mg/L lead and arsenic.

** RPD= relative percent difference.

*** The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM)

7.0 Chain-of-Custody Procedures

All media once received by the Laboratory must be maintained under standard chain-of-custody.

8.0 Data Handling and Verification

All sample weights, fluid concentrations ,and calculations must be recorded on data sheets. Finally all key data will be entered into the attached EXCEL spreadsheet for final delivery and calculation of relative bioavailability.

9.0 References

Casteel, S.W., R.P. Cowart, C.P. Weis, G.M. Henningsen, E.Hoffman and J.W. Drexler, 1997. Bioavailability of lead in soil from the Smuggler Mountain site of Aspen Colorado. Fund. Appl. Toxicol. 36: 177-187.

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methods, and quality assurance, ASTM STP 1226, M.E. Beard and S.A. Iske (Eds.). American Society for Testing and Materials, Philadelphia, PA, 19103-1187.

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Phone: (303) 492-5251

The In-Vitro Method
Relative Bioavailability Leaching Procedure
By
J.W. Drexler, Ph.D.
University of Colorado

I made the following change from the original method.

I. Sample preparation (section 4.0 of the method)

I sieved the soil sample first to obtain the desire grain size of Φ <250mm. Then the sample was dried at 105°C for 1 hour to remove all water molecules and have the exact sample weight. Before collect the 1.00 ± 0.5g of sample (dried soil), the sample was transferred to the desiccators containers until the sample temperature drop to room temperature (this to avoid water absorption from the surrounding environment) for at least 45 to 1.0 hour.

I made some tests in the extraction fluid preparation in where I prepared the extraction fluid and added the hydrochloric acid without warm (37.0 ± 2.0 °C) the extraction fluid and the buffers. When I compared with the extraction solution prepared according the original method not significant changes was observed.

I ran both water tank units (BLP apparatus) at the same time and took me almost 2.5 hours to filter all the samples (20 samples) and the method said that the total time to filter and take pH reading is 1.5 hours. To avoid that I ran both water tank units with 1.0 thru 1.5 hours of difference, in that manner I had enough time to filter and take pH readings without pass the 1.5 hours threshold.

The In-Vitro Method
Bioavailability Leaching Procedure
BLP

1.0 Purpose

The purpose of this method is to determinate what fraction of a contaminant (in this case lead (Pb^{2+})) is absorbed by an organism via a specific exposure route (in this case orally). Several researchers have developed in vitro tests to measure the fraction of a chemical present in a soil sample under simulated gastrointestinal conditions. This test consisted of an aqueous fluid (solution, mainly simulate gastric juices present in our gastrointestinal tract) into which the contaminant is introduced. Once this procedure is complete, the solution is analyzed. The mass of the lead found in the filtered extract is compared to the mass introduced into the test. The fraction liberated into the aqueous phase is defined as the bio-available fraction of lead in that media. To date, for lead-bearing materials tested in the USEPA swine studies, this in vitro assay has correlated well ($R2 = 0.93$, $p= 0.0001$) with relative bioavailability.

2.0 Sample Preparation

Take the soil samples and sieve it to <250mm grain size, this is used because this is the particle size is representative of that adheres to children's hands. Place the sieved soil samples in an oven at 105°C for 1 hour; then, transfer it to a desiccator containers from 45 min thru 1.0 hour to decrease the sample temperature and to avoid water molecules re-absorption. Samples were thoroughly mixed prior to use to ensure homogenization. Samples are archived after the study completion and retained for further analysis for a period of six months unless otherwise requested. Prior to obtaining a sub-sample for testing in this procedure, each sample must be homogenized in its sample container by end-over-end mixing.

3.0 Apparatus and Materials

3.1 Equipment

The main piece of equipment required for this procedure is the extraction device illustrated in the figure 1. The device holds ten 125ml wide mouth high-density polyethylene (HDPE) bottles. These are rotated within a Plexiglas tank by a TCLP extractor motor with a modified flywheel. The water bath must be filled such that the extraction bottles remained immersed. Temperature in the water bath is maintained at 37 ± 2°C using an immersion circulator heater (Fisher Scientific Model 730).

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This method uses an aqueous extraction fluid at a pH value of 1.5. This fluid is prepared as follows:

Prepare 2L of aqueous extraction fluid using ASTM Type II de-ionized Water (DIW). The buffer is made up in the following manner. To 1.90L of DI water, add 60.06g of Glycine (free base, reagent grade) and bring the solution volume to 2.0L (0.4M Glycine). Place the mixture in the water bath at 37°C until the extraction fluid reaches 37°C. Standardize the pH meter (one should use both a 2.0 and a 4.0 pH buffers for standardization) using temperature compensation at 37°C or buffers maintained at 37°C in the water bath. Add trace metal grade, concentrated hydrochloric acid (12.1N) until the solution pH reaches a value of 1.50 ± 0.05 (approximately 60.0ml).

All reagents must be free of lead and arsenic, and the final fluid must be tested to confirm that lead and arsenic concentrations are less than one-fourth the project required detection limits (PRDLs) of 10 and 20 µg/L, respectively (e.g. less than 2 µg/L lead and 5µg/L arsenic in the final fluid. Cleanliness of all materials used to prepare and /or store the extraction fluid and buffer is essential. All glassware and equipment used to prepare standards and reagents must be properly cleaned, acid washed and finally, triple-rinsed with DI water prior to use.

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Add 1.00 ± 0.5g of test substrate (<250mm soil sample) to the bottle, ensuring that static electricity does not cause soil particles to adhere the lip or outside threads of the bottle. If necessary, use an antistatic brush to eliminate static electricity prior to adding the media. Record the mass of substrate (soil sample) and add the spiking standard solution to those samples that you want to spike. When is ready to begin the test, measure 100 ± 0.5 ml of the extraction fluid, using a graduated cylinder or auto pipette and transfer to the 125ml wide mouth HDPE bottles. Hand-tighten each bottle top and shake/invert to ensure that no leakage occurs, and that no media is caked on the bottom of the bottle. Place the bottle into the modified TCLP extractor, making sure each bottle is secure and the lid(s) are tightly fastened. Fill the extractor with 125 ml bottles containing test materials or QA samples. The temperature of the water bath must be 37 +/- 2 °C. Turn on the extractor and rotate end-over-end at 30 +/- 2 rpm for 1 hour.

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